

# Glycosylation and thermodynamic versus kinetic stability of horseradish peroxidase

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**Abstract** The influence of *N*-linked glycans on the stability of glycoproteins has been studied using horseradish peroxidase isoenzyme C (HRP), which contains eight asparagine-linked glycans. HRP was deglycosylated (d-HRP) with trifluoromethanesulfonic acid and purified to an enzymatically active homogeneous protein containing (GlcNAc)<sub>2</sub> glycans. The thermal stability of HRP and d-HRP at pH 6.0, measured by residual activity, was indistinguishable and showed transition midpoints at 57°C, whereas the unfolding in guanidinium chloride at pH 7.0, 23°C was 2–3-fold faster for d-HRP than for HRP. The results are compatible with a glycan-induced decrease in the dynamic fluctuation of the polypeptide chain.

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**Key words:** Glycoprotein stability; Glycoprotein unfolding; Horseradish peroxidase; Thermodynamic stability; Kinetic stability

## 1. Introduction

The assignment of specific biological roles to the glycans of glycoproteins is a major focus of current work in glycobiology. In this context the fundamental physico-chemical properties of the bulky glycans have been seen as increasingly important [1–3]. Several studies have addressed the stabilizing effect of glycans but reached conflicting conclusions. Some find no stabilizing effect [4–7], whereas other workers report a positive contribution to stabilization due to glycans [8–10]. In the present communication, we demonstrate that the thermodynamic stability of horseradish peroxidase isoenzyme C (HRP) in equilibrium studies at various temperatures is indistinguishable from that of chemically deglycosylated HRP (d-HRP), whereas the unfolding rate constant of d-HRP in guanidinium chloride (GdmCl), and hence the kinetic stability, decreases 2–3-fold.

HRP contains a heme group, two calcium ions [11,12] and eight asparagine-linked glycans. The most abundant glycan, Man $\alpha$ 3(Man $\alpha$ 6)(Xyl $\beta$ 2)Man $\beta$ 4GlcNAc $\beta$ 4(Fuc $\alpha$ 3)GlcNAc-, is typical of plant glycoproteins [13]. The *M<sub>r</sub>* of the enzyme is 44 000 as determined by sequence analysis and electrospray mass spectrometry [14,15]. Peroxidases are particularly suited for glycoprotein stability and folding studies, as the heme group can be used as a sensitive probe of the native and the unfolded states ( $N \leftrightarrow U$ ) using absorption spectrometry. In

addition, the structurally stabilizing calcium ions can be trapped with EDTA on unfolding, thus making this process practically irreversible [16].

## 2. Materials and methods

Lyophilized HRP was from Mann Research Laboratories Inc. (lot U1929, RZ 3.1) (used for the original sequence analysis [14], and comparable to Sigma type VI). Deglycosylated HRP (d-HRP) [17] was prepared using trifluoromethanesulfonic acid in the presence of phenol at –10°C, and purified by benzhydroxamic acid affinity and ion exchange chromatography, yielding enzymatically active homogeneous d-HRP of *M<sub>r</sub>* 35 500 which contained only (GlcNAc)<sub>2</sub> glycans. The *pI* values of d-HRP and HRP were both 9, and the polypeptide chains were identical in all respects [17].

All reagents were analytical grade and from Sigma. Buffers were made with water purified on a Waters Milli-Q system.

Peroxidase activity was measured with 0.1 mM H<sub>2</sub>O<sub>2</sub> and 0.34 mM *o*-dianisidine in 50 mM sodium acetate, pH 5.3, at room temperature. The reaction was followed by observing the increase in absorbance at 460 nm.

Absorption spectra and spectral changes were recorded on a Beckman DU 70 spectrophotometer fitted with a thermostatted cuvette. Peroxidase unfolding was initiated by adding the native protein to the cuvette containing GdmCl, EDTA and buffer at pH 7.0, 23°C. The reaction was followed by observing the decrease in absorbance of the Soret band of the native peroxidases at 402 nm (*A*), which decreases as the heme is released. The concentration of native peroxidase at time *t* over the initial concentration at time 0,  $[\text{native}]_t/[\text{native}]_0 = (A_t - A_\infty)/(A_0 - A_\infty)$ . At 402 nm  $\epsilon = 100 \text{ mM}^{-1} \text{ cm}^{-1}$  for native and  $40 \text{ mM}^{-1} \text{ cm}^{-1}$  for unfolded peroxidase.

## 3. Results

### 3.1. Thermal stability

Samples of HRP and d-HRP were incubated at different temperatures and the thermal equilibrium between the native and unfolded forms arrested by trapping released structural calcium ions using 20 mM EDTA at room temperature, solution conditions which can be assumed to prevent further unfolding and refolding (Fig. 1). In the absence of EDTA, the thermal equilibrium curves moved markedly toward higher temperatures. The residual activity is therefore a measure of the amount of the native peroxidase present at the various temperatures. The 20 mM EDTA had no effect on the activity of peroxidase at room temperature. Furthermore, no indicators of irreversible unfolding such as aggregation were observed. Fig. 1 shows that no difference exists between the thermal stability of HRP and d-HRP within experimental error. The transition midpoints are at 57°C.

### 3.2. Unfolding in GdmCl

HRP is stable in 8 M urea at room temperature and the more potent denaturant GdmCl was therefore used to compare the unfolding of HRP and d-HRP. The unfolding of

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**Abbreviations:** GdmCl, guanidinium chloride; G\*, standard molar transition state free energy; G°, standard molar free energy; HRP, horseradish peroxidase isoenzyme C; d-HRP, deglycosylated homogeneous HRP

HRP and d-HRP in GdmCl in the presence of 20 mM EDTA is irreversible due to the binding of calcium ions by EDTA [16]. A semi-logarithmic plot of the experimental data can be fitted to a straight line indicating that the unfolding obeys first-order kinetics and is unimolecular (Fig. 2). The rate constant for the unfolding process from native to unfolded protein ( $N \rightarrow U$ )  $k_u = -\ln([native]_t/[native]_0)/t$  is equal to the negative slope. At 5.2 M GdmCl  $k_u = 0.50 \pm 0.01 \times 10^{-3} \text{ s}^{-1}$  for HRP and  $1.6 \pm 0.1 \times 10^{-3} \text{ s}^{-1}$  for d-HRP. At 5.8 M GdmCl  $k_u = 2.0 \pm 0.1 \times 10^{-3} \text{ s}^{-1}$  for HRP and  $4.2 \pm 0.1 \times 10^{-3} \text{ s}^{-1}$  for d-HRP. The fully glycosylated HRP is 2–3-fold more stable than deglycosylated d-HRP, depending on GdmCl concentration, in this irreversible kinetic experiment. The difference in transition state free energy of unfolding,  $\Delta\Delta G^*_u = -RT\Delta\ln(k_u)$ , between HRP and d-HRP is approximately 3 kJ/mol in 5.2 M GdmCl and 2 kJ/mol in 5.8 M GdmCl. This need for a higher energy input to unfold HRP is consistent with reduced structural fluctuations in the protein induced by large glycans.

#### 4. Discussion

The polypeptide chains of wild-type HRP ( $M_r = 44\,000$ ;  $pI = 9$ ) and the trifluoromethanesulfonic acid deglycosylated d-HRP ( $M_r = 35\,500$ ;  $pI = 9$ ) used in the present study are identical [17]. Furthermore, the glycans of HRP appear to belong to the flexible protein surface shielding type analyzed extensively in ribonuclease [3], as all eight asparagine side chains bound to the glycans of plant HRP are exposed on the surface [12], and non-glycosylated HRP expressed in *Escherichia coli* is able to fold into a fully competent enzyme [18]. An NMR study comparing the dynamics of non-glycosylated ribonuclease A and various glycoforms of ribonuclease B by amide proton exchange showed that “glycosylation decreased dynamic fluctuations throughout the molecule” [19]. This result suggests that a large glycan and its associated solvent molecules dampen movements within the associated protein domain and that, furthermore, the higher transition state energy which is needed for unfolding will be attained less frequently. This is precisely what is observed by the 2–3-fold slower unfolding rate of d-HRP compared with HRP, depending on GdmCl concentration. Considering the backward re-

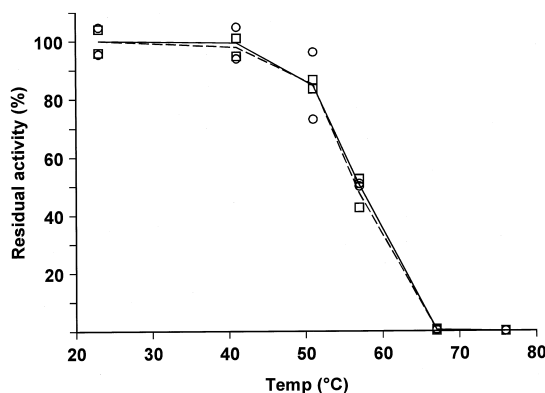


Fig. 1. Heat stability of HRP and d-HRP. The peroxidases, HRP (○, solid line) and d-HRP (□, dashed line), at 1.1  $\mu\text{M}$  concentration were incubated for 15 min at different temperatures in 50 mM sodium citrate buffer titrated with HAc to pH 6.0, and then quickly diluted 20-fold with 20 mM EDTA at room temperature before measuring the residual activity.

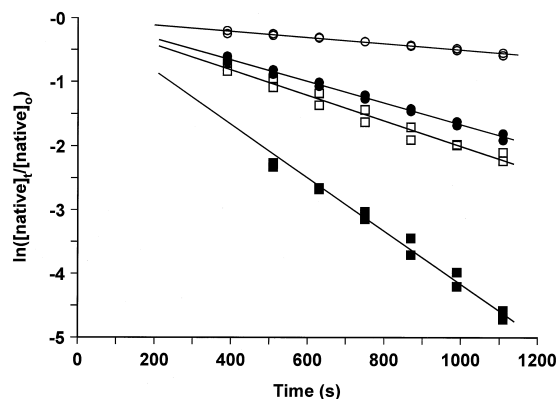


Fig. 2. Unfolding of HRP and d-HRP in GdmCl. The unfolding of peroxidase (2.3  $\mu\text{M}$  or 0.1 mg/ml) in GdmCl, 20 mM EDTA, 0.05 M bisTrisCl, pH 7.0, 23°C was observed as the decrease in absorption at 402 nm. This decrease is coincident with the decrease in peroxidase activity [16]. The  $\ln([native]_t/[native]_0)$  is plotted versus time for HRP (○) and d-HRP (●) at 5.2 M GdmCl, and for HRP (□) and d-HRP (■) at 5.8 M GdmCl.

action in a reversible experiment, a comparable dampening of the dynamics of the unfolded glycosylated protein which will reduce the rate of refolding, appears equally reasonable. As the equilibrium constant between the native and unfolded forms ( $N \leftrightarrow U$ )  $K = k_u/k_f$ , which is the ratio of the rate constants of unfolding ( $k_u$ ) and folding ( $k_f$ ), it is evident that  $K$ , and hence the thermodynamic stability  $\Delta G^\circ = -RT\ln K$  will depend very little on the extent of glycosylation, as observed for HRP and d-HRP.

In the present study the thermal stability at near reversible conditions is compared to the irreversible unfolding in GdmCl. This comparison is only valid if the mechanisms of unfolding and refolding by temperature and GdmCl are comparable. This is presumably the case, as the mechanisms of the heat and urea reactions were similar in a previous study of unfolding and refolding of the homologous fungal peroxidase from *Coprinus cinereus*, but different from the mechanism of unfolding and refolding at alkaline pH [16].

#### 5. Conclusions

In most practical applications, the unfolding rate of a protein (or its kinetic stability) is the essential stability parameter, as the unfolded form can be easily modified (degraded by proteases, chemically modified or aggregated) making the folding process partially irreversible, and thus making the thermodynamic stability observed at reversible conditions a less relevant parameter.

In the case of HRP, which carries eight *N*-linked glycans of the flexible surface type, these glycans hardly affected the thermodynamic stability, yet increased the kinetic stability significantly. The study stresses furthermore that the degree of reversibility of experimental stability studies is essential to the results obtained.

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